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14. ABSTRACT
Studies were performed to determine the influence of the phosphodiesterase-5 inhibitor, sildenafil, on sensitivity to Adriamyin (doxorubicin) in four human breast tumor cell lines and one murine breast tumor line. Sildenafil did not interfere with the effectiveness of Adriamycin in any of the cell lines tested. Sildenafil also failed to protect MDA-MB231 cells against the cytotoxicity of cisplatin, taxol or camptothecin. Sildenafil enhanced sensitivity to Adriamycin markedly in the p53 mutant MDA-MB231 and p53 null MCF-7/E6 cells and moderately in the MCF-7/caspase 3 and 4T1 cell lines. In the MDA-MB231 cells, sildenafil increased the extent of DNA damage induced by Adriamycin as well as the extent of apoptotic cell death. Sildenafil did not influence sensitivity to Adriamycin in bone marrow cells or macrophages. In an immunocompetent model of breast cancer (4T1 mammary carcinoma in Balb/c mice), sildenafil did not attenuate the antitumor effects of Adriamycin; furthermore, the combination of sildenafil with Adriamycin was no more toxic to the animals than Adriamycin alone. Given that sildenafil has been shown to have the potential to protect the heart against the toxicity of Adriamycin, these studies suggest that the inclusion of sildenafil with conventional chemotherapeutic protocols involving Adriamycin (and possibly cisplatin, camptothecin and/or taxol) should not compromise the antitumor effectiveness of these drugs nor enhance their toxicity to the patient. (taken from our recently published paper).

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Adriamycin; sildenafil; breast cancer; 4T1; apoptosis

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INTRODUCTION

Our proposed work was based on the observations that phosphodiesterase-5 inhibitors, specifically erectile dysfunction drugs such as sildenafil, could protect cardiomyocytes and the heart from the toxicity of the anthracycline antibiotic, adriamycin (1). Furthermore, we had generated preliminary data that sildenafil did not appear to protect breast tumor cells from adriamycin and, in fact, may have promoted sensitivity to this drug. Our research goals were to extend these observations and to delineate the mechanistic basis for these differential effects in the tumor and in the heart.

The attached paper (2), recently published in the journal Breast Cancer Research and Treatment, represents the culmination of our studies. Essentially, we have determined that sildenafil does not interfere with the effectiveness of chemotherapeutic drugs both in cell culture and in an in vivo model of breast cancer in immunocompetent animals. The cell culture studies suggested that sildenafil might actually enhance the response to adriamycin; while the in vivo studies slightly support this observation, the difference in response to adriamycin and adriamycin + sildenafil in the animal model did not reach statistical significance, and therefore we cannot conclusively say whether this might, in fact, be the case. It is possible that continued treatment with sildenafil after adriamycin or the use of a phosphodiesterase-5 inhibitor with different pharmacokinetics might have demonstrated potentiation to the antitumor effects of adriamycin. Nevertheless, assuming the findings relating to the cardioprotective actions of sildenafil can be confirmed in clinical trials, our work indicates that there should not be concerns that sildenafil might somehow attenuate or interfere with the effectiveness of chemotherapy against breast cancer.

We did not pursue the signaling elements relating to nitric oxide and nitric oxide synthase in this work. This was due to the fact that we were unable to confirm the findings of the Fisher report (1) relating to these signaling pathways using cardiomyocytes in culture. This may have been a consequence, in part, of the fact that we were using a different experimental model system of adriamycin cardiotoxicity than the Fisher paper (1). Nevertheless, the H9c2 model is well accepted in the literature as an indication of adriamycin-induced damage to the heart (3,4).

CONTRACTUAL ISSUES AND TECHNICAL ISSUES (in response to Final Report Review)

Our primary efforts during the final year of work were devoted to completing the studies in the appended manuscript and submitting this work for publication. A significant period of time was spent in developing the animal model system in our laboratory.

With regard to the question relating to the xenograft model (MDA-MB2321 cells) indicated in the statement of work. We chose to modify our approach and to perform experiments with a rodent tumor model system (4T1 mouse mammary tumor cells) rather than the xenograft model. The rationale for this choice was that studies in vivo with mouse breast tumor cells permit the utilization of an immunocompetent animal, which is more physiologically similar to the environment associated with drug treatment in the patient than the xenograft (a human tumor line in a mouse). The fact that the 4T1 mouse breast tumor cells responded to the combination of sildenafil + adriamycin in the same manner as the MDA-MB231 cells made this modified protocol feasible.

BODY

Data presented in previous reports relating to studies in cardiomyocytes

In terms of exploring the mechanisms whereby sildenafil might protect cardiomyocytes while allowing adriamycin to target the breast tumor cell, it is important to recognize that the foundation for the differential effects is likely to be doxorubicin acting as a topoisomerase II poison in tumor cells (5) while its toxicity to the heart is thought to be through the generation of free radicals (3,4). However, our studies raise some questions relating to this paradigm.

The experimental model system for these studies has been the H9c2 cardiomyocyte cell line. This is an embryonic cell line that replicates in culture and which has been used by a number of investigators as a model of cardiac function (6,7).

Sildenafil failed to protect the cardiomyocytes against the impact of adriamycin in a clonogenic survival assay. Furthermore, sildenafil also failed to protect the cardiomyocytes from apoptosis. These studies raised some questions as to the validity of the findings relating to sildenafil cardioprotection in rodent models, although clearly an intact animal is likely to be a more physiologically relevant model that cardiomyocytes in cell culture.

Adriamycin generated reactive oxygen in the cardiomyocyte. However, while N-acetyl cysteine, a scavenger of free radicals, clearly reduced the extent of reactive oxygen generation, it failed to protect against the toxicity of adriamycin. In contrast, NAC was quite effective in protecting the cardiomyocytes against the toxicity of H_2O_2. These findings raise questions relating to the basis for adriamycin cardiotoxicity, questions that are supported by recent work suggesting that free radicals may not fully account for adriamycin-induced injury to the heart (4,8).

Studies in Breast Tumor Cells

The attached PDF of our recently published paper (2) summarizes the outcomes of our studies combining chemotherapy and sildenafil in breast tumor cell models both in cell culture and in vivo. The Figure numbers below refer to the Figures in this paper.

The influence of sildenafil on sensitivity to Adriamycin was initially evaluated in three isogenic MCF-7 human breast tumor cell lines and in MDA-MB231 breast tumor cells using a standard MTT dye assay. Figure 1 shows the effects of treatment with various concentrations of Adriamycin either in the absence or presence of 10 μM sildenafil, the concentration that was shown to protect cardiac myocytes from Adriamycin (1). MCF-7 cells are wild type in p53; MCF-7/caspase 3 cells were engineered to express the executioner caspase, caspase-3, which is not expressed in wild type MCF-7 cells, and MCF-7/E6 cells have p53 function abrogated by the viral E6 protein. MDA-MB231 cells are mutant in p53, represent a triple negative breast tumor cell line, and are frequently used as a model of metastatic breast cancer.

In all cases, sildenafil failed to protect the breast tumor cells against the antiproliferative/cytotoxic actions of Adriamycin. No effect of sildenafil on sensitivity to Adriamycin was evident in the MCF-7 cells; however, sildenafil appeared to increase the extent

of growth inhibition by Adriamycin in the MCF-7/caspase 3 cells, MCF-7/E6, and MDA-MB231 cells. The lack of protection and selective enhancement of sensitivity to Adriamycin by sildenafil was confirmed in a clonogenic survival assay (Figure 2). Sildenafil had no impact on the response to Adriamycin in MCF-7 cells, an observation that is consistent with the findings using the MTT dye assay presented in Fig. 1. There was a relatively modest potentiation of Adriamycin sensitivity in MCF-7/caspase 3 cells, and marked potentiation of sensitivity to Adriamycin in the p53 mutant MDA-MB231 cells and in the MCF-7/E6 cells .

Since the most pronounced effects of sildenafil on sensitivity to Adriamycin in the clonogenic assays were observed in the p53 mutant MDA-MB231 cells, we further evaluated the effect of sildenafil on sensitivity of MDA-MB231 cells to three other antitumor drugs that are used in the treatment of breast cancer, specifically paclitaxel (Taxol), camptothecin and cisplatin. As shown in Fig. 3, sildenafil itself produced a modest but statistically significant suppression of cell growth and viability. Sildenafil did not protect the MDA-MB231 cells from the cytotoxic/antiproliferative effects of these agents but did produce a detectable increase in sensitivity to cisplatin.

We also assessed the extent of DNA damage induced by Adriamycin in MDA-MB231 cells in the absence and presence of sildenafil treatment. Studies of 53BP-1 immunofluorescence and γ-H2AX phosphorylation are presented in Fig. 4a–d. Sildenafil unequivocally enhanced the phosphorylation of γ-H2AX and the expression of 53BP-1. The TUNEL assay provided further evidence of drug potentiation as the extent of apoptosis induced by Adriamycin was clearly increased by pretreatment of the MDA-MB231 cells with sildenafil. In similar studies in MCF-7 cells, we did not observe any increases in either the phosphorylation of γ-H2AX, 53BP-1 immunofluorescence or apoptosis, the latter by cell cycle analysis for detection of sub-G1 cells (data not shown); these findings are consistent with the failure of sildenafil to enhance sensitivity to Adriamycin in MCF-7 cells.

The cardiotoxic actions of Adriamycin have been associated primarily with the generation of reactive oxygen species (9), and the cardioprotective actions of sildenafil are associated, in part, with suppression of free radical generation in the heart (10). Since data in the literature also support a free radical component of Adriamycin action in tumor cells (11), it was of interest to determine whether sildenafil could suppress the generation of reactive oxygen species in breast tumor cells. MCF-7 cells were pretreated with 10 μM of sildenafil for 1 h followed by exposure to 200 μM of hydrogen peroxide for 1 h, and cell viability was monitored by trypan blue exclusion as this assay generally provides a relatively rapid read out of gross cellular injury. Figure 5a shows that hydrogen peroxide suppressed cell growth (data shown at days 1 and 3) and that the free radical scavenger, *N*-acetyl cysteine, protects against the growth suppressive effects of hydrogen peroxide; however, sildenafil was ineffective in this regard. In support of these findings, Fig. 5b demonstrates that *N*-acetyl cysteine reduced reactive oxygen generation by hydrogen peroxide (by DCF staining) but that sildenafil again failed to suppress reactive oxygen/free radical generation.

Based on these studies as well as those reported by Fisher et al. (1), it is reasonable to suggest that sildenafil may have the potential to protect the heart against the cardiotoxicity of Adriamycin without interfering with the antitumor action of Adriamycin or other conventional agents that are utilized to treat breast cancer. However, since Adriamycin (like a number of other

antitumor drugs) is also immunosuppressive (12), it was also relevant to examine the interaction of sildenafil with Adriamycin in experimental models of bone marrow and macrophage viability.

Figure 6a, b shows that Adriamycin altered the cell cycle distribution of bone marrow cells, producing a marked reduction in both the G1 and G2/M populations while increasing the sub G1/G0 population that is indicative of apoptosis; sildenafil did not attenuate, enhance, or otherwise modify the influence of Adriamycin on cell cycle distribution or apoptosis in these cells. The studies presented in Fig. 6c, d support these findings by demonstrating that sildenafil fails to either suppress or exacerbate Adriamycin-induced apoptosis (assessed by the TUNEL assay) in the bone marrow cells

Figure 7a presents the results of studies of the effects of Adriamycin alone and Adriamycin plus sildenafil on viability of macrophages. Adriamycin clearly suppressed the growth of the macrophages; however, there was no additional effect of sildenafil, either enhancement of sensitivity to Adriamycin or protection from Adriamycin-induced apoptosis in studies performed at both 2 and 48 h after the initial exposures. Again, with regard to assessing the potential free radical actions of Adriamycin, we evaluated whether the free radical scavengers, glutathione, and *N*-acetyl cysteine could protect macrophages against the cytotoxicity of Adriamycin. Figure 7b indicates that *N*-acetyl cysteine produced a modest protection at 48 h; similar results were evident with glutathione (data not shown). In contrast, NAC and GSH treatment resulted in a quite pronounced protection against the toxicity of hydrogen peroxide (Fig. 7b). It was furthermore of interest that sildenafil failed to attenuate the antiproliferative/cytotoxic activity of hydrogen peroxide, indicating that sildenafil does not function in macrophages in a manner similar to its putative mode of action in the heart.

While these findings suggesting that sildenafil does not protect breast tumor cells against the effects of chemotherapy (and, in some cases, enhances the impact of chemotherapy) are encouraging, it remains to be determined whether these observations are also relevant in vivo. For these purposes, studies were performed using the 4T1 murine mammary carcinoma cells in immunocompetent syngeneic Balb/c mice, a well-established preclinical system for evaluating drug action against breast cancer (13). Prior to initiating the animal studies, we assessed the impact of sildenafil on sensitivity to Adriamycin in 4T1 cells in culture. Figure 8a indicates that sildenafil produced a modest enhancement of Adriamycin sensitivity.

Studies in vivo were performed with two 10 mg/kg doses of Adriamycin administered at days 1 and 7 with each dose preceded by 0.7 mg/kg of sildenafil. Figure 8b indicates that sildenafil did not interfere with the antiproliferative action of Adriamycin; a slight, though barely significant enhancement of Adriamycin activity was detected. Figure 8c demonstrates that an essentially identical profile of weight loss was evident with Adriamycin alone and Adriamycin plus sildenafil, indicating that the inclusion of sildenafil did not increase overall toxicity.

KEY RESEARCH ACCOMPLISHMENTS (Taken from Abstract from recently published paper)

1. Sildenafil did not interfere with the effectiveness of Adriamycin in any of the breast tumor cell lines tested.

2. Sildenafil also failed to protect MDA-MB231 cells against the cytotoxicity of cisplatin, taxol or camptothecin.

3. Sildenafil enhanced sensitivity to Adriamycin markedly in the p53 mutant MDA-MB231 and p53 null MCF-7/E6 cells and moderately in the MCF-7/caspase 3 and 4T1 cell lines.

4. In the MDA-MB231 cells, sildenafil increased the extent of DNA damage induced by Adriamycin as well as the extent of apoptotic cell death.

5. Sildenafil did not influence sensitivity to Adriamycin in bone marrow cells or macrophages.

6. In an immunocompetent model of breast cancer (4T1 mammary carcinoma in Balb/c mice), sildenafil did not attenuate the antitumor effects of Adriamycin.

7. The combination of sildenafil with Adriamycin was no more toxic to the animals than Adriamycin alone.

REPORTABLE OUTCOMES (entire period of grant support)

Di X, Newsham I, Shiu R and Gewirtz DA. Apoptosis, autophagy and accelerated senescence in the response of human breast tumor cells to Adriamycin. Biochem. Pharmacol. 77(7):1139-50. 2009.

Di X, Bright AT, Bellott R, Gaskins E, Robert J, Holt S, Gewirtz D, Elmore L. A chemotherapy-associated senescence bystander effect in breast cancer cells. Cancer Biol Ther. 2008. 7(6):864-872.

Gewirtz D, Holt SE and Elmore LW. Accelerated senescence: An emerging role in tumor cell response to chemotherapy and radiation. Biochemical Pharmacology 2008. 76(8): 947-957.

Gewirtz DA. Autophagy, Senescence and Tumor Dormancy in Cancer Chemotherapy. Commentary. Autophagy. 2009;5(8):1232-4.

Di X, Gennings C, Bear HD, Graham LJ, Sheth CM, White KL Jr, Gewirtz DA. Influence of the phosphodiesterase-5 inhibitor, sildenafil, on sensitivity to chemotherapy in breast tumor cells. Breast Cancer Res Treat. 2010 Feb 13. [Epub ahead of print]

Xu Di received his PhD degree in Pharmacology and Toxicology (June 2009).

Submission of IDEA Expansion Grant to the DOD in 2010 relating to irradiation induced cardiac damage during breast cancer treatment.

Submission of IDEA Grant to the DOD in 2010 relating to irradiation induced cardiac damage during breast cancer treatment.

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Gerald DeMasters (now PhD, MD)
Xu Di (now PhD)
Chris Gennings, PhD, Professor of Biostatistics
Eden Wilson (PhD candidate)
David A. Gewirtz, Professor of Pharmacology and Toxicology

CONCLUSIONS

1. Sildenafil fails to protect breast tumor cells from adriamycin based on multiple assays (viable cell number, clonogenic survival, cell cycle distribution and DNA damage). The clonogenic survival assays support the conclusion that sildenafil appears to sensitize breast tumor cells lacking functional p53 to Adriamycin. Since breast cancer frequently is shown to express mutant p53, these findings support the potential utility of sildenafil as a cardioprotectant that is unlikely to interfere with the antitumor actions of Adriamycin (or other chemotherapeutic agents; see details and data in previous reports).

2. Sildenafil does not increase Adriamycin toxicity to bone marrow cells or macrophages. Again, these findings indicate that sildenafil is unlikely to increase host toxicity of Adriamycin.

3. Adriamycin has the capacity to produce multiple modes of cell death in the breast tumor cells. We now have evidence that Adriamycin also functions through the generation of free radicals to promote senescence. These findings raise questions relating to the selectivity of Adriamycin against the tumor cell versus the heart.

4. Sildenafil does not interfere with the antitumor effects of Adriamycin in an animal model of breast cancer.

5. Inclusion of sildenafil with conventional chemotherapeutic protocols involving Adriamycin (and possibly cisplatin, camptothecin and/or taxol) should not compromise the antitumor effectiveness of these drugs nor enhance their toxicity to the patient.

REFERENCES

1. Fisher P.W., Salloum F., Das A., Hyder H., and Kukreja R.C. Phosphodiesterase-5 inhibition with sildenafil attenuates cardiomyocyte apoptosis and left ventricular dysfunction in a chronic model of doxorubicin cardiotoxicity. *Circulation.*111: 1601-1610, 2005.

2. Di X, Gennings C, Bear HD, Graham LJ, Sheth CM, White KL Jr, Gewirtz DA. Influence of the phosphodiesterase-5 inhibitor, sildenafil, on sensitivity to chemotherapy in breast tumor cells. *Breast Cancer Res Treat.* Feb 13, 2010. [Epub ahead of print]

3. Gilleron M, Marechal X, Montaigne D, Franczak J, Neviere R, Lancel S. NADPH oxidase participates to doxorubicin induced cardiac myocyte apoptosis. *Biochem Biophys Res Commun*. 388(4):727-31, 2009.

4. Shi R, Huang CC, Aronstam RS, Ercal N, Martin A, Huang YW. N-acetyl cysteine amide decreases oxidative stress but not cell death induced by doxorubicin in H9c2 cardiomyocytes. *BMC Pharmacol.* 9:7, 2009.

5. Gewirtz DA. Commentary: A critical analysis of the mechanisms of action proposed for the anthracycline antibiotics, adriamycin and daunorubicin in the tumor cell. *Biochem Pharmacol.* 57: 727-741, 1999.

6. Salas-Vidal E., Lomeli H., Castro-Obregon S., Cuervo R., Escalante-Alcalde D. and Covarrubias L. Reactive oxygen species participate in the control of mouse embryonic cell death. *Exp Cell Res* 238: 136–147, 1998.

7. Kimes B. W., and B. L. Brandt. Properties of a clonal muscle cell line from rat heart. *Exp. Cell Res.* 98: 367-381, 1976.

8. Kobayashi S, Volden P, Timm D, Mao K, Xu X, Liang Q. Transcription factor GATA-4 inhibits doxorubicin induced autophagy and cardiomyocyte death. *J Biol Chem.* 285(1):793-804, 2010.

9. Minotti G et al (2004) Anthracyclines: molecular advances and pharmacologic developments in antitumor activity and cardiotoxicity. *Pharmacol Rev.* 56(2):185–229, 2004.

10. Das A, Xi L, Kukreja RC Phosphodiesterase-5 inhibitor sildenafil preconditions adult cardiac myocytes against necrosis and apoptosis: essential role of nitric oxide signaling. *J Biol Chem* 280(13):12944–12955, 2005.

11. Kurz EU, Douglas P, Lees-Miller SP Doxorubicin activates ATM-dependent phosphorylation of multiple downstream targets in part through the generation of reactive oxygen species. *J Biol Chem* 279(51):53272–53281, 2004.

12. Wijayahadi NH, Haron MR, Stanslas J, Yusuf Z Changes in cellular immunity during chemotherapy for primary breast cancer with anthracycline regimens. *J Chemother* 19(6):716–723, 2007.

13. Laginha KM et al.Determination of doxorubicin levels in whole tumor and tumor nuclei in murine breast cancer tumors. *Clin Cancer Res* 11(19):6944–6949, 2005.

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PRECLINICAL STUDY

Influence of the phosphodiesterase-5 inhibitor, sildenafil, on sensitivity to chemotherapy in breast tumor cells

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Abstract Studies were performed to determine the influence of the phosphodiesterase-5 inhibitor, sildenafil, on sensitivity to Adriamycin (doxorubicin) in four human breast tumor cell lines and one murine breast tumor line. Sildenafil did not interfere with the effectiveness of Adriamycin in any of the cell lines tested. Sildenafil also failed to protect MDA-MB231 cells against the cytotoxicity of cisplatin, taxol or camptothecin. Sildenafil enhanced sensitivity to Adriamycin markedly in the p53 mutant MDA-MB231 and p53 null MCF-7/E6 cells and moderately in the MCF-7/caspase 3 and 4T1 cell lines. In the MDA-MB231 cells, sildenafil increased the extent of DNA damage induced by Adriamycin as well as the extent of apoptotic cell death. Sildenafil did not influence sensitivity to Adriamycin in bone marrow cells or macrophages. In an immunocompetent model of breast cancer (4T1 mammary carcinoma in Balb/c mice), sildenafil did not attenuate the antitumor effects of Adriamycin; furthermore, the combination of sildenafil with Adriamycin was no more toxic to the animals than Adriamycin alone. Given that sildenafil has been shown to have the potential to protect the heart against the toxicity of Adriamycin, these studies suggest that the inclusion of sildenafil with conventional chemotherapeutic protocols involving Adriamycin (and possibly cisplatin, camptothecin and/or paclitaxel) should not compromise the antitumor effectiveness of these drugs nor enhance their toxicity to the patient.

Keywords Sildenafil · Cardiotoxicity · Breast cancer · Adriamycin (doxorubicin) · Chemotherapy

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Introduction

Treatment of breast cancer patients (as well as patients with other malignancies) using conventional chemotherapeutic drugs is almost uniformly accompanied by a spectrum of host toxicities, some of which may be life threatening, such as suppression of bone marrow function. One of the dose-limiting toxicities of Adriamycin (doxorubicin), a drug frequently utilized for the treatment of breast cancer as well as other malignancies [1], is a cumulative cardiomyopathy that is associated with morbidity and mortality even after many years from the cessation of treatment [1, 2]. Other drugs and treatments that may compromise cardiac function, either alone or in combination, include trastuzumab, ionizing radiation as well as tyrosine kinase-targeting drugs [2–4]. Currently, one of the few agents used clinically for cardioprotection (principally against

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doxorubicin) is dexrazoxane (ICRF187) [5]. Unfortunately, dexrazoxane may itself produce immunosuppression and can also increase Adriamycin-induced hematotoxicity, such as leukopania [6]. Another major drawback of dexrazoxane is its relatively high cost. Consequently, a pressing need exists for alternative cardioprotectants that are minimally toxic to the patient.

Phosphodiesterase-5 inhibitors including sildenafil, tadalafil, and vardenafil are drugs that have now been utilized for a number of years for the treatment of erectile dysfunction. Sildenafil and tadalafil have also been shown to protect against cardiac ischemia [7]. In addition, studies have shown that one of these agents, sildenafil, has the potential to protect the heart against the cardiotoxicity of Adriamycin [8].

The possibility of developing cardioprotectants from drugs that have been prescribed for millions of patients with little evidence of toxic side effects is quite attractive. However, before this option can be considered, it is necessary to establish that this class of agents does not concurrently protect tumor cells from chemotherapy. Given that Adriamycin is one of the primary drugs used in the treatment of breast cancer, the current studies were designed to assess the influence of the phosphodiesterase-5 inhibitor, sildenafil, on the antiproliferative and cytotoxic effects of Adriamycin in breast cancer cells. The impact of sildenafil on sensitivity of breast tumor cells to a number of additional agents conventionally utilized in the treatment of breast cancer was also examined. We also evaluated whether sildenafil might enhance the toxicity of Adriamycin to bone marrow cells and macrophages. Finally, the effect of sildenafil on the response to Adriamycin was investigated in an immunocompetent model system, 4T1 mammary carcinoma growing in Balb/C mice.

Materials and methods

Materials

RPMI 1640 medium with l-glutamine, trypsin–EDTA (0.05% trypsin, 0.53 mM EDTA-4 Na), penicillin/streptomycin (10,000 units/ml penicillin and 10 mg/ml streptomycin), and fetal bovine serum were obtained from Invitrogen (Eugene, OR). Defined bovine calf serum was obtained from Hyclone Laboratories (Logan, UT). Reagents used for the TUNEL assay (terminal transferase, reaction buffer, and Fluorescein-dUTP) were purchased from Roche Diagnostics Corporation (Indianapolis, IN). X-gal was obtained from Gold Biotechnology (St. Louis, MO). The following materials were obtained from Sigma Chemical (St. Louis, MO): formaldehyde, acetic acid, albumin bovine (BSA), lactic acid, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), 2′,7′-dichlorofluorescein diacetate (DCF-DA), *N*-acetyl-l-cysteine (NAC), reduced glutathione (GSH), 6-diamidino-2-phenylindole (DAPI), and dimethyl sulfoxide (DMSO). Acridine orange was purchased from Molecular Probes (Eugene, OR). Adriamycin, camptothecin, paclitaxel, and cisplatin were obtained from Sigma Chemical Company, St. Louis, MO. Antibodies for γ-H2AX were obtained from Upstate Biotechnology Inc (Lake Placid, NY). The 53BP1 antibody was kindly provided by Dr. Lynne Elmore. Anti-mouse IgG was obtained from KPL Inc. (Gaithersburg, MD). Anti-β-actin antibody was purchased from Sigma Chemical Company.

Cell culture

The MCF-7 and MDA-MB231 breast tumor cell lines were obtained from the NCI Frederick Cancer Research Facility. The isogenic cell line, MCF-7/E6, was established by stable retroviral infection as described previously [9]. The MCF-7 cells expressing caspase 3 were described in previous studies [10]. 4T1 mouse mammary carcinoma cells were purchased from The American Type Culture Collection (ATCC, Manassas, VA). Cells were maintained as monolayer cultures in RPMI 1640 media supplemented with glutamine (0.292 mg/ml), penicillin/streptomycin (0.5 ml/100 ml media), and 10% fetal bovine serum. Cells were cultured at 37°C in 5% CO_2 and 100% humidity. Cells were exposed to Adriamycin for various periods as indicated with or without pretreatment with 10 μM sildenafil for 1 h. In all cases, sildenafil was maintained in the medium throughout the course of the experiment.

Assessment of viable cell number

Cell viability was determined by trypan blue exclusion at various time points beginning 24 or 72 h after initiation of sildenafil or Adriamycin treatment. Cells were harvested by trypsinization, stained with 0.4% trypan blue dye, and counted using phase contrast microscopy. Cells that excluded trypan blue dye were considered to be viable.

Effects on sildenafil on sensitivity to Adriamycin by the MTT assay

In order to determine the effects of sildenafil on sensitivity to Adriamycin in MCF-7, MDA-MB231, MCF-7/caspase 3, and MCF-7/E6 cells, cells were seeded in triplicate wells at 8000 cells per well of a 96-well cluster plate and were pretreated with 10 μM sildenafil for 1 h followed by continuous exposure to Adriamycin. At 72-h post-drug exposure, cell viability was assessed using a standard MTT assay. This involved adding 100 μl of 2 mg/ml MTT per well, incubating in the dark for 3 h, carefully removing the MTT, and then adding 100 μl DMSO per well. Absorbance

was measured at 490 nm with an EL800 Universal Microplate Reader (Bio-Tek Instruments Inc.).

TUNEL assay for apoptosis

The method of Gavrieli et al. [11] was utilized as an independent assessment of apoptotic cell death in combined cytospins containing both adherent and non-adherent cells, as reported previously [12]. Cells were fixed, and the fragmented DNA in cells undergoing apoptosis was detected using the In Situ Cell Death Detection Kit (Roche), where strand breaks are end labeled with fluorescein dUTP by the enzyme terminal transferase. TUNEL positive cells were quantitated by counting the number of positive cells per field using Image-Pro Plus software by Media Cybernetics, L.P. Three representative fields were averaged per condition.

Clonogenic survival assay

Cells were plated in triplicate in six-well tissue culture dishes at the appropriate density for each condition and treated with Adriamycin, sildenafil, or Adriamycin plus sildenafil. After 14 days, the cells were fixed with 100% methanol, air-dried for 1–2 days and stained with 0.1% crystal violet. For computing the survival fraction, groups of 50 or more cells were counted as colonies. Data were normalized relative to untreated controls that were taken as 100% survival.

Propidium iodide staining and flow cytometry

Cell cycle analysis was performed using prodidium iodide staining and flow cytometry. After treatments with Adriamycin, sildenafil, or sildenafil plus Adriamycin, cells were harvested using trypsin, pelleted by centrifugation, and washed twice with PBS. Cellular DNA was labeled by resuspending 1×10^6 cells in 1 ml propidium iodide staining solution (3.8 mM NaCitrate, 0.05 mg/ml propidium iodide, 0.1% triton X-100, 9 K units/ml RNase B). Before analysis, each sample was filtered through a 37-μm nylon mesh. Nuclei were analyzed with the Guava EasyCyte^{TM} Mini System (Hayward, CA).

ROS detection by DCF-DA staining

Dichlorofluorescein diacetate (DCF-DA) was used to detect reactive oxygen species (ROS) [13] following exposure to sildenafil and Adriamycin. MCF-7 cells were seeded in 6-well plates and treated as described in the cell viability study above. At the appropriate times after treatment, cells were incubated for 30 min with medium containing 5 μM DCF-DA. After 30 min, medium containing DCF-DA was removed, and fresh medium was added to the flasks. Fluorescence was immediately visualized with an inverted fluorescent microscope.

Western blotting

Cells were lysed in 60 mM Tris (pH 6.8) containing 2% SDS and a cocktail of protease inhibitors (Sigma Chemical Company) at the indicated time points. Whole cell lysates were boiled for 5 min, briefly sonicated, and then centrifuged for 10 min at 10,000 × *g* at 4°C. Protein concentrations were determined using a Lowry-based spectrophotometric assay (BioRad, Hercules, CA), according to the manufacturer's protocol. From each sample, 10–20 μg was separated by SDS-PAGE and electrotransferred onto nitrocellulose membrane. A standard blotting procedure was performed using monoclonal antibodies directed against γ-H2AX (1:1,000) followed by peroxidase-conjugated anti-mouse IgG (1:10,000). In order to control for protein loading, all membranes were subsequently probed with a β-actin antibody (1:2,000).

Detection of 53BP1 foci by immunohistochemistry

MCF-7 cells were seeded in eight-well chamber slides 24 h before drug or radiation exposure. At the appropriate times after sildenafil or Adriamycin treatments, cells were rinsed twice with PBS and then fixed with 3.7% paraformaldehyde in PBS for 10 min at room temperature. Cells were washed twice more with PBS and permeabilized in 0.5% NP40 in PBS for 10 min at room temperature. After two more washings with PBS, cells were blocked for 30 min in PBS with gelatin. The chamber slide basket was then removed before incubation with primary antibody. 53BP1 primary antibody was added to cells, and slides were covered with parafilm. After overnight incubation at 4°C, slides were washed 3 × 5 min with PBS. The slides were then incubated with fluorescein-conjugated secondary antibody in PBS with gelatin for 1 h. Following this 1 h of incubation, slides were washed 3 × 5 min with PBS. Vectashield with DAPI was added to slides, and coverslips were placed over cells. 53BP1 foci were visualized using an inverted fluorescent microscope.

Bone marrow cell isolation

Bone marrow was isolated from the femurs and tibias of nude mice. The bone marrow was triturated using a 22-gauge needle to make a single cell suspension in 1× PBS. The bone marrow cells were centrifuged at 1,000 rpm for 3 min and washed twice with 1× PBS. Red blood cell lysis buffer was added to the cells at 37° C for 5 min and cells were washed twice in 1× PBS. Finally, bone marrow cells were resuspended and cultured in IMDM media

supplemented with 30% FBS. Cells were plated for TUNEL assay and flow cytometry analysis within 24 h.

Preparation of macrophages

Thioglycollate-elicited peritoneal macrophages (TEPMs) were obtained by injecting mice intraperitoneally with 1 ml 10% Brewer's yeast thioglycollate. Four days later, cells were harvested from each mouse via peritoneal lavage with 8 ml of sodium bicarbonate buffered Hank's Balanced Salt Solution (HBSS) and aspiration of the lavage fluid into a 10-cc syringe. The lavage fluids containing TEPMs from groups of six mice, were pooled into a 50-ml conical tube. Following centrifugation, the pellets were resuspended in RPMI 1640 supplemented with 10% fetal calf serum and penicillin [100 U/ml]/streptomycin [100 μg/ml].

Studies in Balb/c mice

4T1 tumor cells (5×10^4 cells suspended in 50 μl PBS) were injected subcutaneously into the right flank of 7-week-old NCI BALB/c mice. The mice were maintained in a pathogen-free environment and tumor size was monitored every 2–3 days. When the tumors reached a size of 200–400 mm^3, mice were randomized into one of four experimental groups (5 per group) and treatment was initiated according to the indicated schedule/dose. Vehicle (saline), sildenafil (0.7 mg/kg), Adriamycin (5 mg/kg), or sildenafil plus Adriamycin was administered intraperitoneally on days 1 and 7 after grouping. Tumor volume was calculated using the following formula: tumor volume (mm^3) = length × width × (1/2 of the greater of length or width). Mouse weight was measured every 2–3 days subsequent to the first injection.

Statistical analysis for in vitro and in vivo studies

Statistical differences were determined using Statview statistical software. A one-way analysis of variance (ANOVA) followed by Tukey test was used to compare the difference between the Adriamycin treatment groups and the sildenafil plus Adriamycin groups. P values ≤ 0.05 were taken as statistically significant.

Statistical analysis for effects on tumor growth

Results of tumor growth in vivo are presented as the means ± standard errors (SE) of tumor volume in each treatment group. Tumor volume was modeled using a nonlinear exponential model as a function of the four treatment groups (control, adriamycin alone, sildenafil alone, and adriamycin with sildenafil). Parameterization of a response surface model was assumed for the three-dimensional relationship between ADR (x_1), SID (x_2), and time (t) using a loglinear model. For fixed levels of ADR and SID, the model is loglinear with time; the effects of ADR, SID, and the interaction were evaluated by comparison of the corresponding parameters in the four loglinear models over time where a common intercept was assumed. Important statistical hypotheses that were tested included:

1. A test of the significance of the tumor growth rate over time in the control group;
2. A test of whether the tumor growth rate over time (control group) changes with treatment of ADR;
3. A test of whether the tumor growth rate over time (control group) changes with treatment of SID; and
4. A test of interaction between the two drugs on tumor growth rate.

The model assumed a linear random effect term to account for intrasubject-correlated observations assuming a compound symmetric correlation structure.

A mixed-effects nonlinear logistic regression model was fit to the adjusted mouse weights allowing for a different slope parameter per treatment group. The nonlinear model accommodated the mean adjusted weight to be greater than 100% and less than 100% with the following parameterization:

$$\mu_i = \frac{\alpha}{1 + \exp(-(\beta_{0i} + \beta_i t))}, \quad i = 1, \ldots, 4$$

The model assumed:

- a fixed value for the plateau parameter, α as estimated from a nonlinear logistic model (i.e., $\alpha = 108$);
- a common intercept for the control group and the sildenafil group; and
- an additive random effect which accommodated a compound symmetric intra-subject-correlation structure.

Both models were estimated using PROC NLMIXED in SAS (version 9.2).

Results

The influence of sildenafil on sensitivity to Adriamycin was initially evaluated in three isogenic MCF-7 human breast tumor cell lines and in MDA-MB231 breast tumor cells using a standard MTT dye assay. Figure 1 shows the effects of treatment with various concentrations of Adriamycin either in the absence or presence of 10 μM sildenafil, the concentration that was shown to protect cardiac myocytes from Adriamycin [7] in MCF-7, MCF-7/caspase 3, MCF-7/E6, and MDA-MB231 cells. MCF-7 cells are wild type in p53; MCF-7/caspase 3 cells were engineered to express the executioner caspase, caspase-3, which is not

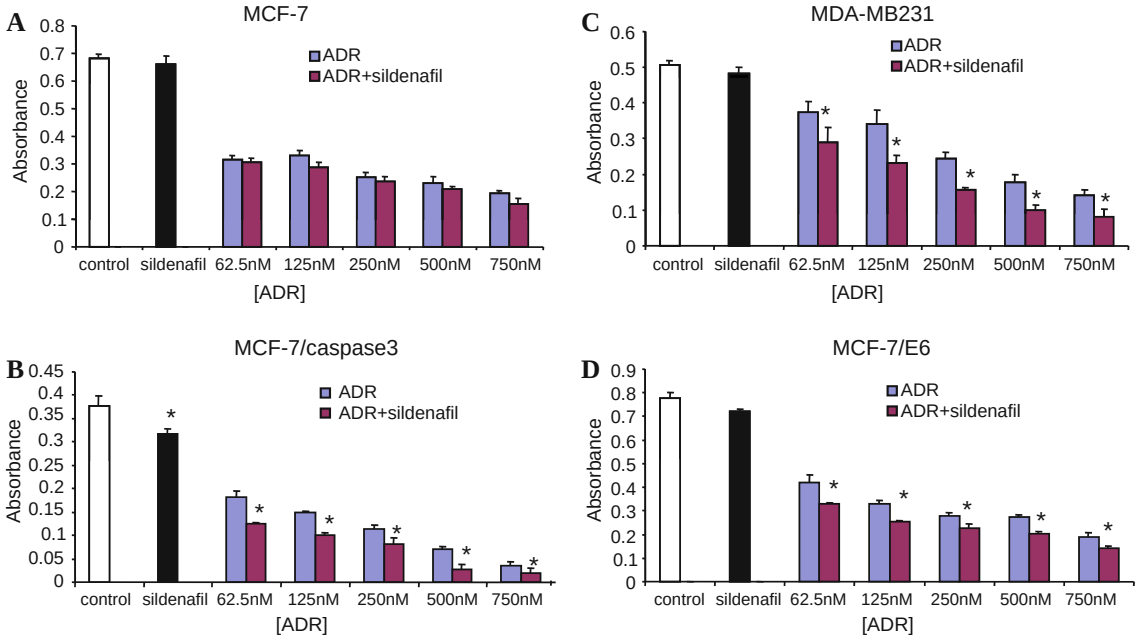


Fig. 1 Influence of sildenafil on sensitivity to Adriamycin in breast tumor cells (MTT assay). MCF-7, MCF-7/caspase 3, MDA-MB231 and MCF-7/E6 cells were treated with 10 μM sildenafil for 1 h prior to exposure to various concentrations of Adriamycin for 72 h. Absorbance on the *y*-axis is an indication of cell number. * $P \leq 0.05$

expressed in wild type MCF-7 cells [10, 14]; and MCF-7/E6 cells have p53 function abrogated by the viral E6 protein [9]. MDA-MB231 cells are mutant in p53, represent a triple negative breast tumor cell line [15], and are frequently used as a model of metastatic breast cancer [16].

In all cases, sildenafil failed to protect the breast tumor cells against the antiproliferative/cytotoxic actions of Adriamycin. No effect of sildenafil on sensitivity to Adriamycin was evident in the MCF-7 cells; however, sildenafil appeared to increase the extent of growth inhibition by Adriamycin in the MCF-7/caspase 3 cells, MCF-7/E6, and MDA-MB231 cells. The lack of protection and selective enhancement of sensitivity to Adriamycin by sildenafil was confirmed in a clonogenic survival assay since clonogenic survival assays are generally considered to be the "gold standard" for assessment of chemosensitivity and radiosensitivity. An Adriamycin concentration of 1 μM and a 2-h exposure time were chosen for these experiments as these conditions tend to simulate the clinical pharmacokinetic profile of the drug after pulse exposure [17]. Figure 2 indicates that sildenafil had no impact on the response to Adriamycin in MCF-7 cells (Fig. 2a), an observation that is consistent with the findings using the MTT dye assay presented in Fig. 1. There was a relatively modest potentiation of Adriamycin sensitivity in MCF-7/caspase 3 cells (Fig. 2b) and marked potentiation of sensitivity to Adriamycin in the p53 mutant MDA-MB231 cells (Fig. 2c), and in the MCF-7/E6 cells (Fig. 2d).

Since the most pronounced effects of sildenafil on sensitivity to Adriamycin in the clonogenic assays were observed in the p53 mutant MDA-MB231 cells, we further evaluated the effect of sildenafil on sensitivity of MDA-MB231 cells to three other antitumor drugs that are used in the treatment of breast cancer, specifically paclitaxel (Taxol), camptothecin and cisplatin. As shown in Fig. 3, sildenafil itself produced a modest but statistically significant suppression of cell growth and viability. Sildenafil did not protect the MDA-MB231 cells from the cytotoxic/antiproliferative effects of these agents but did produce a detectable increase in sensitivity to cisplatin.

Since Adriamycin acts primarily through the induction of DNA damage by the inhibition of topoisomerase II [17], we assessed the extent of DNA damage induced by Adriamycin in MDA-MB231 cells in the absence and presence of sildenafil treatment. Studies of 53BP-1 immunofluorescence and γ-H2AX phosphorylation are presented in Fig. 4a–d. Sildenafil unequivocally enhanced the phosphorylation of γ-H2AX and the expression of 53BP-1 (Fig. 4a, b); this finding is consistent with the sensitization to Adriamycin in the MDA-MB231 cells by sildenafil (Figs. 1, 2). The TUNEL assay provided further evidence of drug potentiation as the extent of apoptosis induced by Adriamycin was clearly increased by pretreatment of the MDA-MB231 cells with sildenafil (Fig. 4c, d). In similar studies in MCF-7 cells, we did not observe any increases in either the phosphorylation of γ-H2AX, 53BP-1 immunofluorescence or apoptosis, the latter by cell cycle analysis for detection of sub-G1 cells (data not shown); these findings are consistent with the failure of sildenafil to enhance sensitivity to Adriamycin in MCF-7 cells.

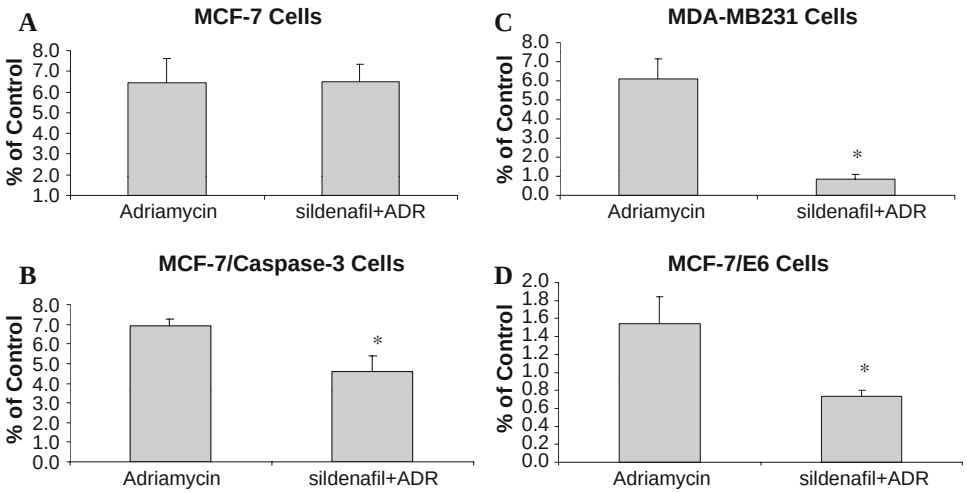
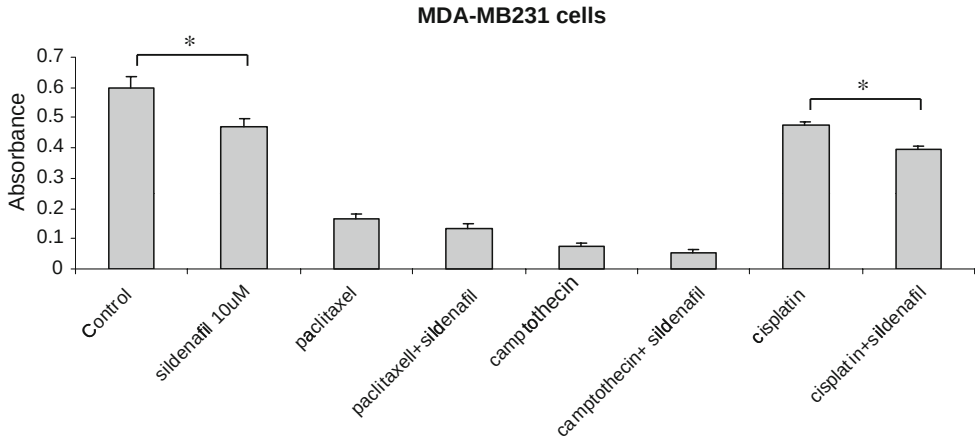


Fig. 2 Influence of sildenafil on sensitivity to Adriamycin in breast tumor cells (Clonogenic survival assay). MCF-7, MCF-7/caspase 3, MDA-MB231 and MCF-7/E6 cells were seeded at different densities (untreated or sildenafil treated cells: 200 cells per dish; Adriamycin or sildenafil plus Adriamycin: 2,000 cells per dish) one day prior to treatment. Cells were treated with 10 μM sildenafil for 1 h prior to exposure to 1 μM Adriamycin for 2 h. A clonogenic survival assay was performed as described in "Materials and methods". * $P \leq 0.05$

Fig. 3 Influence of sildenafil on sensitivity to various chemotherapeutic drugs in MDA-MB231 cells. Cells were pretreated with 10 μM sildenafil for 1 h followed by chronic treatment with 100 nM paclitaxel, 5 μM camptothecin or 5 μM cisplatin. The MTT assay was performed at 72 h as described in "Materials and methods"



The cardiotoxic actions of Adriamycin have been associated primarily with the generation of ROS [1, 2], and the cardioprotective actions of sildenafil are associated, in part, with suppression of free radical generation in the heart [7, 18]. Since data in the literature also support a free radical component of Adriamycin action in tumor cells [19], including our own recent studies relating to Adriamycin induced senescence [20], it was of interest to determine whether sildenafil could suppress the generation of ROS in breast tumor cells. MCF-7 cells were pretreated with 10 μM of sildenafil for 1 h followed by exposure to 200 μM of hydrogen peroxide for 1 h, and cell viability was monitored by trypan blue exclusion as this assay generally provides a relatively rapid read out of gross cellular injury. Figure 5a shows that hydrogen peroxide suppressed cell growth (data shown at days 1 and 3) and that the free radical scavenger, *N*-acetyl cysteine, protects against the growth suppressive effects of hydrogen peroxide; however, sildenafil was ineffective in this regard. In support of these findings, Fig. 5b demonstrates that *N*-acetyl cysteine reduced reactive oxygen generation by hydrogen peroxide (by DCF staining) but that sildenafil again failed to suppress reactive oxygen/free radical generation.

Based on these studies as well as those reported by Fisher et al. [8], it is reasonable to suggest that sildenafil may have the potential to protect the heart against the cardiotoxicity of Adriamycin without interfering with the antitumor action of Adriamycin or other conventional agents that are utilized to treat breast cancer. However, since Adriamycin (like a number of other antitumor drugs) is also immunosuppressive [21], it was also relevant to examine the interaction of sildenafil with Adriamycin in experimental models of bone marrow and macrophage viability.

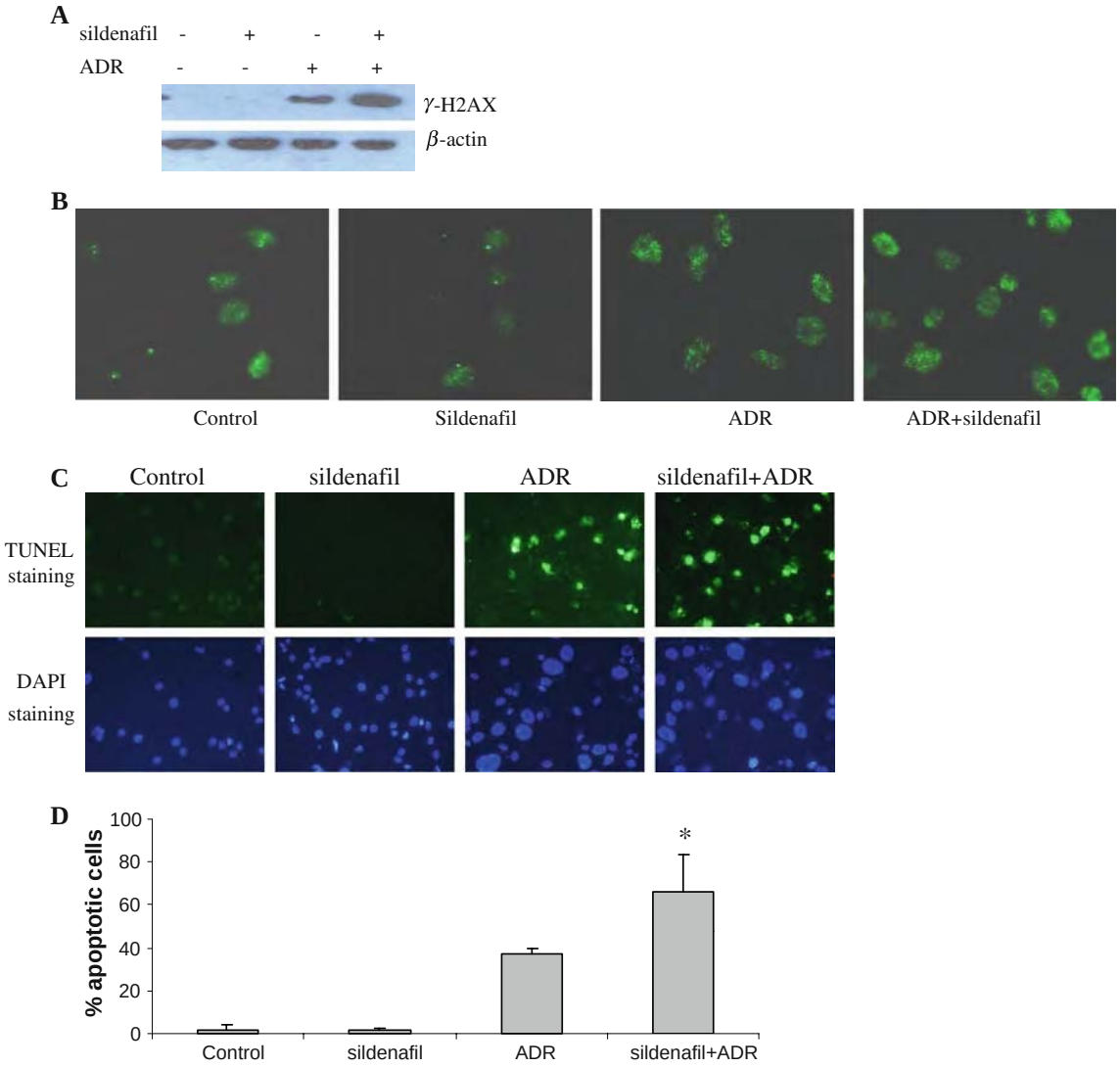


Fig. 4 Influence of sildenafil on Adriamycin-induced DNA damage response in MDA-MB231 cells. Cells were treated with sildenafil, Adriamycin or sildenafil plus Adriamycin as indicated for Fig. 3. Six hours post treatment, the cells were harvested for western blotting or fixed for immuno-fluorescence, respectively. **a** γ-H2AX phosphorylation. **b** 53BP-1 staining. **c** TUNEL staining performed 4 days post Adriamycin or sildenafil treatment. *Upper panels*, TUNEL staining; *lower panels*, DAPI staining. **d** Quantitation of TUNEL assay. * $P \leq 0.05$

Figure 6a, b shows that Adriamycin altered the cell cycle distribution of bone marrow cells, producing a marked reduction in both the G1 and G2/M populations while increasing the sub G1/G0 population that is indicative of apoptosis; sildenafil did not attenuate, enhance, or otherwise modify the influence of Adriamycin on cell cycle distribution or apoptosis in these cells. The studies presented in Fig. 6c, d support these findings by demonstrating that sildenafil fails to either suppress or exacerbate Adriamycin-induced apoptosis (assessed by the TUNEL assay) in the bone marrow cells.

Figure 7a presents the results of studies of the effects of Adriamycin alone and Adriamycin plus sildenafil on viability of macrophages. Adriamycin clearly suppressed the growth of the macrophages; however, there was no additional effect of sildenafil, either enhancement of sensitivity to Adriamycin or protection from Adriamycin-induced apoptosis in studies performed at both 2 and 48 h after the initial exposures. Again, with regard to assessing the potential free radical actions of Adriamycin, we evaluated whether the free radical scavengers, glutathione, and *N*-acetyl cysteine could protect macrophages against the cytotoxicity of Adriamycin. Figure 7b indicates that *N*-acetyl cysteine produced a modest protection at 48 h; similar results were evident with glutathione (data not shown). In contrast, NAC and GSH treatment resulted in a

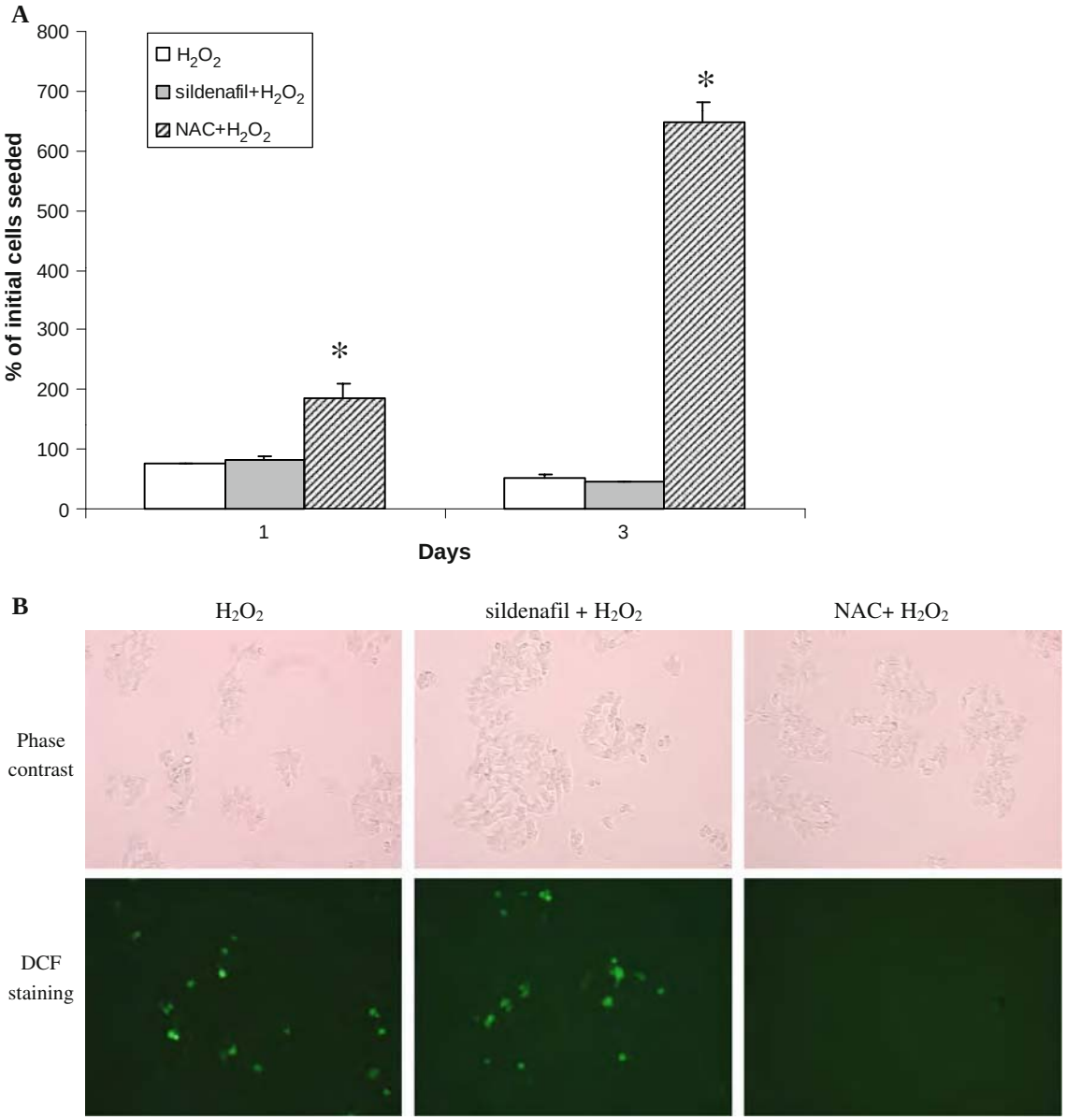


Fig. 5 Sildenafil fails to block hydrogen peroxide induced reactive oxygen species (ROS) generation. MCF-7 cells were pretreated with 10 μM sildenafil or 20 mM *N*-acetyl-cysteine (NAC) for 1 h followed by 200 μM hydrogen peroxide for 1 h. The drugs were removed and fresh media was restored with or without sildenafil. **a** Viable cell number was determined based on trypan blue exclusion on days 1 or 3. Data is plotted in relation to the initial cell number before treatment. **b** DCF-DA staining was performed at 24 h post hydrogen peroxide treatment. * $P \leq 0.05$

quite pronounced protection against the toxicity of hydrogen peroxide (Fig. 7b). It was furthermore of interest that sildenafil failed to attenuate the antiproliferative/cytotoxic activity of hydrogen peroxide, indicating that sildenafil does not function in macrophages in a manner similar to its putative mode of action in the heart.

While these findings suggesting that sildenafil does not protect breast tumor cells against the effects of chemotherapy (and, in some cases, enhances the impact of chemotherapy) are encouraging, it remains to be determined whether these observations are also relevant in vivo. For these purposes, studies were performed using the 4T1 murine mammary carcinoma cells in immunocompetent syngeneic Balb/c mice, a well-established preclinical system for evaluating drug action against breast cancer [22]. Prior to initiating the animal studies, we assessed the impact of sildenafil on sensitivity to Adriamycin in 4T1 cells in culture. Figure 8a indicates that sildenafil produced a modest enhancement of Adriamycin sensitivity.

Studies in vivo were performed with two 10 mg/kg doses of Adriamycin administered at days 1 and 7 with each dose preceded by 0.7 mg/kg of sildenafil. Figure 8b

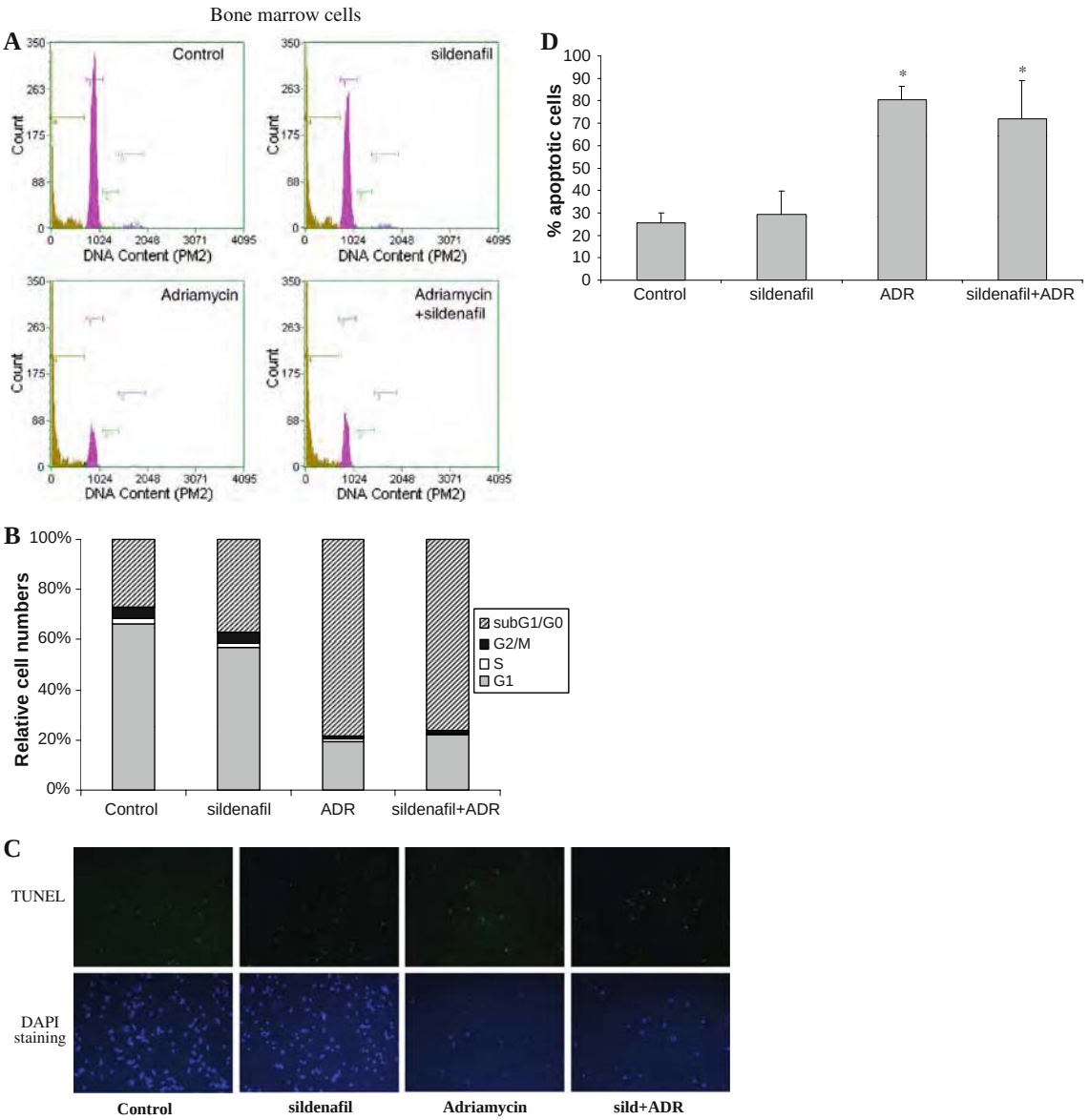


Fig. 6 Lack of influence of sildenafil on Adriamycin-induced apoptosis of bone marrow cells. Bone marrow cells were isolated and seeded as described in "Materials and methods". The cells were continuously treated with 1 μM Adriamycin in the presence or absence of 10 μM sildenafil for 48 h. **a** FACS analysis. **b** Cell cycle distribution. **c** *Upper panels*, TUNEL assay; *lower panels*, DAPI staining. **d** Quantitation of apoptosis. * $P \leq 0.05$

indicates that sildenafil did not interfere with the antiproliferative action of Adriamycin; a slight, though barely significant enhancement of Adriamycin activity was detected. Figure 8c demonstrates that an essentially identical profile of weight loss was evident with Adriamycin alone and Adriamycin plus sildenafil, indicating that the inclusion of sildenafil did not increase overall toxicity.[1]

[1] In a separate study, where the mice received a second dose of Adriamycin of 5 mg/kg, we also observed that sildenafil did not interfere with the antitumor actions of Adriamycin nor exacerbate the weight loss (data not shown). Tumor-bearing mice that were not treated with drug showed no weight loss and essentially maintained their weight after an initial slight weight gain (data not shown).

A more detailed statistical analysis of these observations is provided in the supplementary data section.

Discussion

Anthracyclines are among the most effective anti-tumor drugs for the treatment of a number of malignancies including breast cancer, childhood solid tumors, lymphomas, and leukemias. However, chronic administration of Adriamycin has potentially serious side effects, such as cardiomyopathy and congestive heart failure [1, 2]. In order to address these problems, Dexrazoxane (ICRF187)

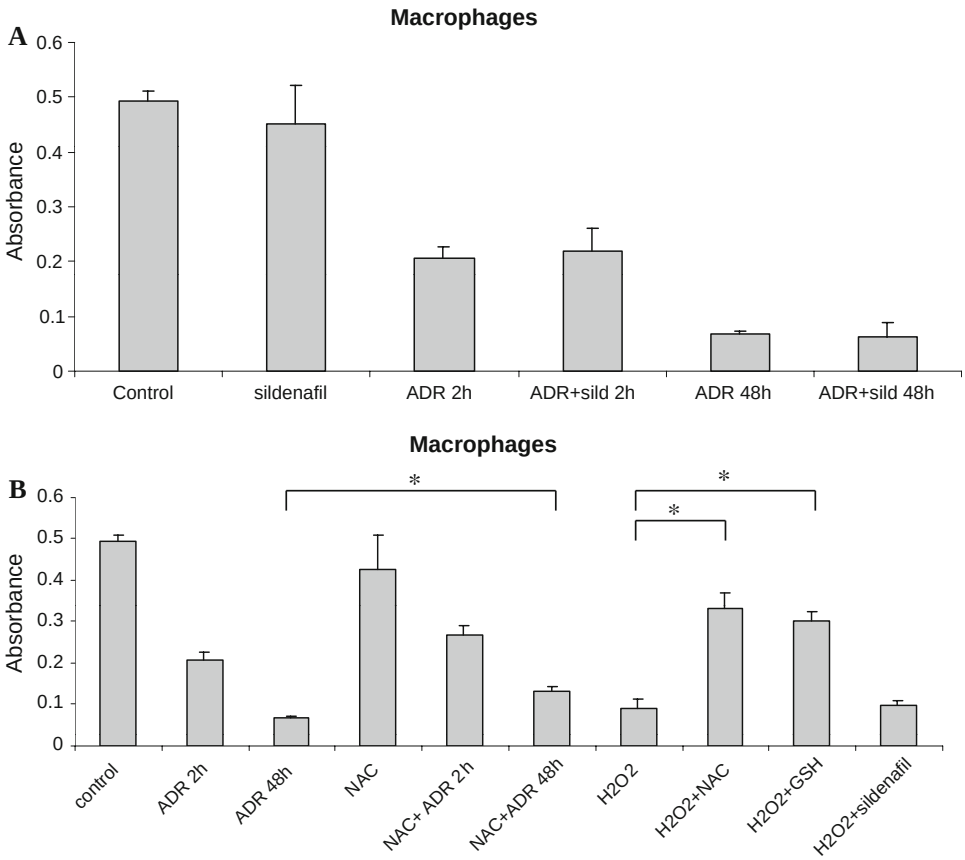


Fig. 7 Influence of sildenafil on sensitivity to Adriamycin in macrophages. Macrophages were prepared as described in "Materials and methods". **a** The macrophages were treated with 1 μM Adriamycin for 2 or 48 h in the presence or absence of 10 μM sildenafil, and the MTT assay was performed 48 h post treatment. **b** Macrophages were treated with 1 μM Adriamycin for 2 h or 200 μM H_2O_2 for 1 h in the presence or absence of 10 mM NAC or GSH. The MTT assay was performed 48 h post treatment

is sometimes used clinically to reduce Adriamycin-induced cardiotoxicity in cancer patients, and presumably acts by preventing iron-based oxygen free-radical generation [23]. However, clinical studies indicate that the addition of dexrazoxane only moderately reduces the risk of symptomatic heart failure, and increases the haematological toxicity associated with chemotherapy [6]. Recognizing these limitations, development of other safer and more effective drugs to prevent anthracycline-induced cardiotoxicity could have value to breast cancer patients as well as other cancer patients treated with this or other cardiotoxic agents.

Sildenafil, a phosphodiesterase-5 inhibitor developed for treatment of erectile dysfunction, has now been shown to have cardioprotective effects in response to simulated ischaemia and reoxygenation [7] as well as Adriamycin-induced heart failure [8] in animal model systems. Our studies were designed to address the critical question of whether sildenafil might also have the ability to protect breast tumor cells against Adriamycin (and/or other antitumor drugs).

In our studies, sildenafil alone had little direct effect on breast tumor cell growth or cell cycle distribution (not shown) at clinically relevant concentrations. Studies of proliferation (assessed by the MTT dye assay) indicated that sildenafil had no cytoprotective actions against Adriamycin-induced toxicity in the four breast cancer cell lines examined and appeared to differentially enhance the antiproliferative effects of the antitumor drug in three of the cell lines (MCF-7/caspase-3, MCF-7/E6, and MDA-MB231 cells). These findings suggest that sensitization to Adriamycin by sildenafil is more likely to occur in the absence of wild type p53 and/or in the presence of functional caspase-3, where the cells are highly susceptible to apoptosis. Studies using a more sensitive clonogenic survival assay confirmed these observations and indicated that the effect of sildenafil on sensitivity to Adriamycin was most pronounced in the p53 mutant MDA-MB231 cells.

The major cause of the toxic effect of Adriamycin in the heart is generally accepted to be an increase in the generation of ROS [1, 2]. Studies have suggested that sildenafil exerts its cardioprotective effects through the suppression of ROS production in both cardiomyocytes culture and mouse models [18, 24]. We have also recently reported that Adriamycin induces a time-dependent increase of ROS in MCF-7 cells [20]. However, while ROS generation by hydrogen peroxide was significantly inhibited by

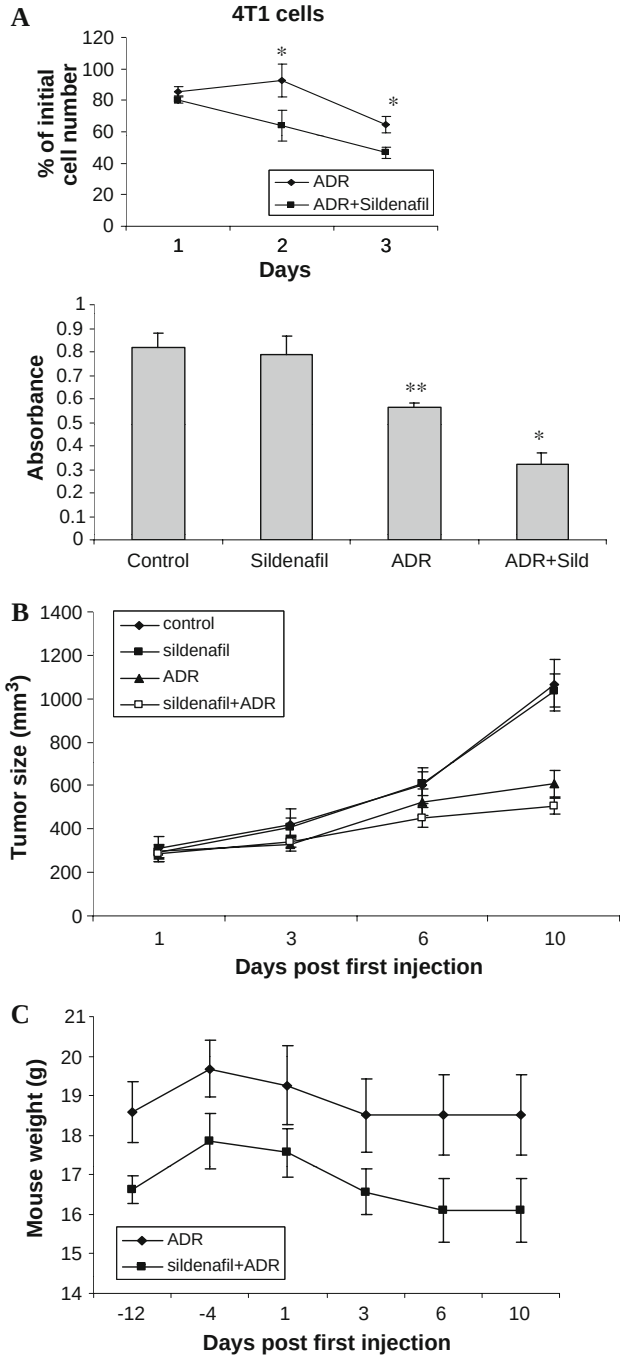


Fig. 8 **a** Influence of sildenafil on sensitivity to Adriamycin in 4T1 breast tumor cells in culture. Cells were exposed to 1 μM of Adriamycin for 2 h preceded by 10 μM sildenafil for 1 h. Viable cell number was determined by trypan blue exclusion (*upper panel*) and drug sensitivity was further assessed by the MTT assay on day 3 (*lower panel*). **b** Impact of sildenafil on growth of 4T1 breast tumor cells in Balbc mice exposed to Adriamycin. **c** Weight of mice in response to treatment. * ADR versus ADR plus sildenafil, $P \leq 0.05$; ** control or sildenafil versus ADR, $P \leq 0.05$

antioxidants, sildenafil failed to suppress ROS generation by hydrogen peroxide, strongly suggesting that sildenafil lacks the capacity to inhibit ROS generation in breast tumor cells.[2] This is consistent with the fact that sildenafil does not protect against Adriamycin in any of the four human breast tumor cell lines or the murine 4T1 breast tumor cell line.

Because DNA damage has been associated with exposure to Adriamycin in cancer cells [25], we further evaluated whether sildenafil could attenuate the Adriamycin-induced DNA damage response. In MDA-MB231 cells, the combined treatment with sildenafil + Adriamycin resulted in a significant enhancement of the DNA damage response (elevated phosphorylation of γ-H2AX and expression of 53BP-1) compared to that for Adriamycin alone. Furthermore, sildenafil increased the extent of apoptosis over that produced by Adriamycin alone. These observations are in dramatic contrast to the suppression of apoptosis by sildenafil in the studies of cardioprotection [7, 8].

In addition to cardiotoxicity, another significant side effect of Adriamycin is immunosuppression, specifically bone marrow depression and macrophage dysfunction [26, 27]. Bone marrow is one of the critical tissues for the generation of immune cells while activation of macrophages is one of the first steps involved in the initiation of an immune response. Since PDE5 is expressed in various cells of the immune system (including macrophages) [28], it was possible that sildenafil might increase Adriamycin toxicity against these cell populations. However, our studies indicate that sildenafil neither promotes nor ameliorates Adriamycin-induced cytotoxicity against either bone marrow cells or macrophages.

Serafini et al. found that sildenafil reversed tumor-induced immunosuppression and enhanced an antitumor response leading to tumor growth delay [29]. While our studies did not detect a significant enhancement of the response to Adriamycin in an immunocompetent mouse model of breast cancer, the fact that sildenafil neither interfered with the anti-tumor effects of Adriamycin nor increased its overall toxicity indicates that sildenafil is unlikely to attenuate the impact of chemotherapeutic agents utilized for the treatment of breast cancer.

In summary, our studies show that sildenafil enhanced sensitivity to Adriamycin in three of four human breast cancer cell lines as well as one murine breast tumor cell line. Moreover, sildenafil did not attenuate the activity of paclitaxel, camptothecin, or cisplatin in the MDA-MB231 breast tumor model. Sildenafil also did not exacerbate Adriamycin toxicity to either bone marrow cells or macrophages. A recent report indicates that PDE5 inhibitors also enhance tumor permeability and efficacy of chemotherapy

[2] It should be noted that it cannot be readily determined whether the reactive species measured reflect peroxide released during apoptosis or residual ROS generated directly from Adriamycin metabolism within the cell.

in a rat brain tumor model [30]. Although we did not observe a significant enhancement of tumor cell responsiveness to Adriamycin in our animal experiments, these studies nevertheless strongly support the potential utility of phosphodiesterase inhibitors such as sildenafil as adjunctive agents for cardioprotection in breast cancer therapy.

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References

1. Minotti G et al (2004) Anthracyclines: molecular advances and pharmacologic developments in antitumor activity and cardiotoxicity. Pharmacol Rev 56(2):185–229
2. Menna P, Salvatorelli E, Minotti G (2008) Cardiotoxicity of antitumor drugs. Chem Res Toxicol 21(5):978–989
3. Garcia-Alvarez A, Garcia-Albeniz X, Esteve J, Rovira M, Bosch X (2009) Cardiotoxicity of tyrosine-kinase-targeting drugs. Cardiovasc Hematol Agents Med Chem
4. Bird BRJH, Swain SM (2008) Cardiac toxicity in breast cancer survivors: review of potential cardiac problems. Clin Cancer Res 14(1):14–24
5. Pouillart P (2004) Evaluating the role of dexrazoxane as a cardioprotectant in cancer patients receiving anthracyclines. Cancer Treat Rev 30(7):643–650
6. Cvetkovi RS, Scott LJ (2005) Dexrazoxane: a review of its use for cardioprotection during anthracycline chemotherapy. Drugs 65:1005–1024
7. Das A, Xi L, Kukreja RC (2005) Phosphodiesterase-5 inhibitor sildenafil preconditions adult cardiac myocytes against necrosis and apoptosis: essential role of nitric oxide signaling. J Biol Chem 280(13):12944–12955
8. Fisher PW et al (2005) Phosphodiesterase-5 Inhibition with sildenafil attenuates cardiomyocyte apoptosis and left ventricular dysfunction in a chronic model of doxorubicin cardiotoxicity. Circulation 111(13):1601–1610
9. Elmore LW et al (2002) Adriamycin-induced senescence in breast tumor cells involves functional p53 and telomere dysfunction. J Biol Chem 277(38):35509–35515
10. Hattangadi DK et al (2004) Influence of p53 and caspase 3 activity on cell death and senescence in response to methotrexate in the breast tumor cell. Biochem Pharmacol 68(9):1699–1708
11. Gavrieli Y, Sherman Y, Ben-Sasson SA (1992) Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation. J Cell Biol 119(3):493–501
12. DeMasters GA et al (2004) Potentiation of cell killing by fractionated radiation and suppression of proliferative recovery in MCF-7 breast tumor cells by the Vitamin D3 analog EB 1089. J Steroid Biochem Mol Biol 92(5):365–374
13. Song YS, Lee BY, Hwang ES (2005) Dinstinct ROS and biochemical profiles in cells undergoing DNA damage-induced senescence and apoptosis. Mech Ageing Dev 126(5):580–590
14. Janicke RU et al (1998) Caspase-3 is required for DNA fragmentation and morphological changes associated with apoptosis. J Biol Chem 273(16):9357–9360
15. Cohen B et al (2009) Cyclin D1 is a direct target of JAG1-mediated Notch signaling in breast cancer. Breast Cancer Res Treat. doi:10.1007/s10549-009-0621-9
16. Ndlovu MN et al (2009) Hyperactivated NF-{kappa}B and AP-1 transcription factors promote highly accessible chromatin and constitutive transcription across the interleukin-6 gene promoter in metastatic breast cancer cells. Mol Cell Biol 29(20):5488–5504
17. Gewirtz DA (1999) A critical evaluation of the mechanisms of action proposed for the antitumor effects of the anthracycline antibiotics Adriamycin and daunorubicin. Biochem Pharmacol 57(7):727–741
18. Fernandes M et al (2008) Sildenafil citrate concentrations not affecting oxidative phosphorylation depress H2O2 generation by rat heart mitochondria. Mol Cell Biochem 309(1):77–85
19. Kurz EU, Douglas P, Lees-Miller SP (2004) Doxorubicin activates ATM-dependent phosphorylation of multiple downstream targets in part through the generation of reactive oxygen species. J Biol Chem 279(51):53272–53281
20. Di X et al (2009) Apoptosis, autophagy, accelerated senescence and reactive oxygen in the response of human breast tumor cells to Adriamycin. Biochem Pharmacol 77(7):1139–1150
21. Wijayahadi NH, Haron MR, Stanslas J, Yusuf Z (2007) Changes in cellular immunity during chemotherapy for primary breast cancer with anthracycline regimens. J Chemother 19(6):716–723
22. Laginha KM et al (2005) Determination of doxorubicin levels in whole tumor and tumor nuclei in murine breast cancer tumors. Clin Cancer Res 11(19):6944–6949
23. Hensley ML et al (2009) American society of clinical oncology 2008 clinical practice guideline update: use of chemotherapy and radiation therapy protectants. J Clin Oncol 27(1):127–145
24. Bivalacqua TJ et al (2009) Sildenafil inhibits superoxide formation and prevents endothelial dysfunction in a mouse model of secondhand smoke induced erectile dysfunction. J Urol 181(2):899–906
25. Banath JP, Olive PL (2003) Expression of phosphorylated histone H2AX as a surrogate of cell killing by drugs that create DNA double-strand breaks. Cancer Res 63(15):4347–4350
26. Asmis R et al (2005) A novel thiol oxidation-based mechanism for adriamycin-induced cell injury in human macrophages. FASEB J 19(13):1866–1888
27. Li W et al (2006) Cell-based assays for profiling activity and safety properties of cancer drugs. J Pharmacol Toxicol Methods 54(3):313–319
28. Essayan DM (2001) Cyclic nucleotide phosphodiesterases. J Allergy Clin Immunol 108(5):671–680
29. Serafini P et al (2006) Phosphodiesterase-5 inhibition augments endogenous antitumor immunity by reducing myeloid-derived suppressor cell function. J Exp Med 203(12):2691–2702
30. Black KL et al (2008) PDE5 inhibitors enhance tumor permeability and efficacy of chemotherapy in a rat brain tumor model. Brain Res 1230:290–302